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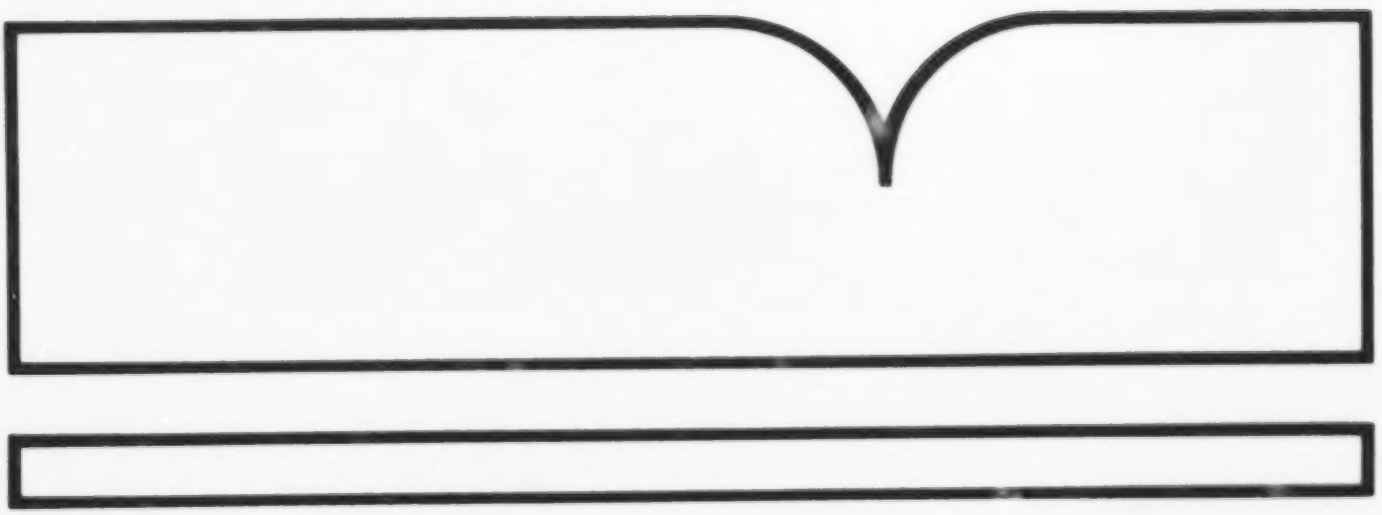

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A NEW METHOD FOR EXCYSTATION OF GIARDIA

Judith F. Sauch

U.S. Environmental Protection Agency

Health Effects Research Laboratory

26 West St. Clair Street

Cincinnati, Ohio 45268

(513) 569-7224

Health Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

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16. ABSTRACT

In vitro excystation of Giardia is used to estimate cyst viability and may also be used to obtain trophozoites and cyst walls for analysis. Recently published excystation procedures include the use of trypsin, serum or bile salts in the "excystation" step. Because G. muris and G. lamblia cyst walls and trophozoites were to be used for antigenic analysis, an alternative method was devised to avoid exposure to trypsin, serum and bile salts. The initial "induction" step of Schaefer was retained, but cyst exposure to the medium was increased to 45 min. Trypsin, serum or bile salts in the "excystation" medium was replaced by proteose peptone for G. muris and phytone peptone for G. lamblia. These reagents were added to Hanks Balanced Salt Solution (HBSS) supplemented with cysteine and sodium bicarbonate. Increased levels of cysteine and glucose in HBSS decreased percent excystation for G. lamblia cysts (from human donors). Comparison between this method utilizing peptones and that of Schaefer utlizing trypsin showed no significant differences in percent excystation for both G. muris and G. lamblia. Variation in percent excystation among seven G. lamblia cyst samples from human donors occurred but the two methods followed similar trends as they varied, with no significant differences between the two. Thus, neither trypsin, serum or bile salts is required for excystation of G. muris or G. lamblia.
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ABSTRACT

In vitro excystation of *Giardia* is used to evaluate cyst viability and may also be used to obtain trophozoites and cyst walls for analysis. Recently published excystation procedures include the use of trypsin, serum or bile salts in the "excystation" step. Because *G. muris* and *G. lamblia* cyst walls and trophozoites were to be used for antigenic analysis, an alternative method was devised to avoid exposure to trypsin, serum and bile salts. An initial acid or low pH "induction" step was retained, but cyst exposure to the medium was increased to 45 min. Trypsin, serum or bile salts in the "excystation" medium was replaced by proteose peptone for *G. muris* and phytone peptone for *G. lamblia*. These reagents were added to Hanks' Balanced Salt Solution (HBSS) supplemented with cysteine and sodium bicarbonate. Increased levels of cysteine and glucose in HBSS decreased percent excystation for *G. lamblia* cysts (from human donors). Comparison between this method utilizing peptones and a method utilizing trypsin showed no significant differences in percent excystation for both *G. muris* and *G. lamblia*. Variation in percent excystation among seven *G. lamblia* cyst samples from human donors occurred but the two methods followed similar trends as they varied, with no significant differences between the two. Thus, neither trypsin, serum or bile salts is required for excystation of *G. muris* or *G. lamblia*.

INTRODUCTION

Currently available excystation procedures expose Giardia cyst walls and trophozoites to complex media containing enzymes, serum, or bile, which might interfere with subsequent electrophoretic analyses. Therefore, alternative reagents were devised in order to avoid exposure to these agents and still obtain high levels of excystation.

Although Hegner concluded in 1927 (6), after observing excystation in vivo, that moisture, temperature, and digestive juices were major factors in the process, it was not until the 1970's that excystation requirements for Giardia were defined more precisely. Bingham and Meyer (2) were the first to demonstrate that although hydrogen ions initiate the excystation process, transfer to a second favorable medium is critical to complete excystation. The "excystation" medium they used (HSP-3;9) contains human serum, phytone, vitamins, amino acids, and salts. Subsequent to these publications, a number of excystation procedures were developed as modifications of the original. In all of them, the acid incubation step is retained for induction, but supplementation of the excystation medium varies. Serum (1,2,4,7), bile (4,7), and enzymes such as trypsin (10,14), pepsin (7,14), and pancreatin (8) have been used with a variety of excystation levels reported. Schaefer et al. (14), however, noted that trypsin, while appearing not to be essential for high levels of excystation of G. muris cysts, seems to accentuate the process.

An alternative excystation medium consisting of balanced salt solution supplemented with a minimum of complex reagents was developed. Since phytone is included in Bingham's medium and appeared to affect excystation favorably (E. W. Rice, personal communication), it was used first to replace serum enzymes and bile. Experiments in which phytone peptone,

proteose peptone, glutathione, cysteine, and glucose were varied or eliminated led to the formulation of a simplified excystation medium consisting of a balanced salts solution supplemented with proteose or phytone peptone, cysteine, and glucose. High excystation levels for G. muris, comparable to published results, and variable, but sometimes high, excystation levels for G. lamblia were obtained. This suggests that complex agents such as enzymes, whole serum, and bile are not essential for Giardia excystation.

MATERIALS AND METHODS

Organisms. G. muris cysts were obtained from female Swiss albino mice (CF-1) infected with cysts per os. Cysts were harvested by modifications of the procedure developed by Roberts-Thomson et al. (11). Mouse feces from approximately 60 animals were pooled after 16-21 hours collection, mixed into a slurry, and diluted to about 2500 ml with 0.01% Tween 20 in distilled water (TDW). After mixing thoroughly, the slurry sat at room temperature for one-half hour to sediment large debris. After this sedimentation was repeated twice, the resulting supernatant fluids were centifuged at 800 x g for 2 minutes. The pellets were repeatedly mixed by vortexing into 0.01% TDW and centrifuged until the supernatant fluids were freed of fine particles. Finally, the pellets were pooled, suspended in about 600 ml TDW, mixed well, dispersed into six round bottom plastic 250 ml centrifuge bottles, underlayed with 80 ml 1.0 M sucrose, and centrifuged in a swinging bucket rotor for 10 minutes at 800 x g. The bands of cysts floating between the sucrose and water layers were collected by aspiration into a flask, diluted 1:5 with TDW, and washed three times in TDW by centrifugation for 5 minutes at 800 x g. Cysts were further purified by sedimentation at unit gravity (12) and stored in distilled water at 2-5°C.

G. lamblia cysts were harvested in a similar manner, with a few modifications, from human donor stool specimens. Large floating particles were filtered out with 2-3 layers of cheesecloth. After washing, pellets were suspended in TDW; 25 ml of the pellet suspension (representing 1 ml of packed pellet volume) was overlayed onto 25 ml Percoll-sucrose, sp. gravity 1.09 (13). Cysts were found in a band between the water and Percoll-sucrose layers. These bands were collected, pooled, diluted 1:5

with TDW and washed three times by centrifugation for 5 minutes at 800 x g. Cysts were purified by sedimentation at unit gravity if more than 10^7 were collected. They were then stored in distilled water at 2-5°C until use.

Excystation of G. lamblia. The acid incubation step (10) was retained. Briefly, 0.5 ml of up to 10^7 cysts in distilled water was transferred to a 15 ml conical centrifuge tube to which was added 5 ml HCl-saline (0.7 ml conc. HCl, 100 ml 0.85% NaCl, pH 1.5), 3 ml reducing solution (HBSS supplemented with 32 mM glutathione and 57 mM L-cysteine-HCl) and 3 ml 0.1 M $NaHCO_3$. The suspension was mixed by vortexing, incubated in a 37°C water bath for 45 minutes, and centrifuged for 2 minutes at 650 x g. The cysts in the pellets were washed once in prewarmed 0.85% NaCl by centrifugation for 2 minutes at 650 x g. The pellet was finally suspended in 1-5 ml prewarmed excystation medium and incubated for 30 minutes in a 37°C water bath. The excystation medium, prepared fresh, was prepared by dissolving 0.015 g $NaHCO_3$ and 0.01 g L-cysteine-HCl monohydrate in 4 ml of 5% phytone peptone and 1 ml 10X HBSS; the final volume was brought to 10 ml with distilled water (final pH 7.1 and final phytone peptone 2%). Stock phytone peptone (5% W/V) was prepared by adding phytone-peptone (BBL #11906) to distilled water and boiling gently with stirring for 10 minutes. After filter sterilization, the stock solution was stored at 2-5°C in small volumes. Samples were removed from the excystation tube and counted in a hemocytometer. At least 200 totally excysted trophozoites (TET), partially excysted trophozoites (PET), and intact cysts (IC) were counted. Excystation was quantitated according to the formula reported by Bingham et al. (3) as follows: [TET ÷ 2 + PET] ÷ [TET ÷ 2 + PET + IC] x 100. In contrast to G. muris, empty

cyst walls of G. lamblia are difficult to observe; however, G. lamblia trophozoites settle quickly and can be counted (10). The number of TET's is halved because every excysted cyst yields a pair of trophozoites (3). This method was compared to that of Rice and Schaefer (11) using G. lamblia cysts isolated from several asymptomatic and symptomatic human donors.

Excystation of G. muris. The induction step was carried out as reported by Schaefer et al. (14) with minor modifications. Between 10^6 and 3 x 10^7 cysts were suspended in 1 ml distilled water in a plastic conical bottom centrifuge tube to which was added 20 ml of reducing solution [Hanks' Balanced salt solution (HBSS) supplemented with 32 mM glutathione and 57 mM L-cysteine HCl] and 20 ml of 0.1 M $NaHCO_3$ (final pH 4.7). The suspension was mixed by vortexing, incubated in a 37°C water bath for 30 minutes, centrifuged at 650 x g for 2 minutes, and then washed once in the excystation medium by centrifugation at 650 x g for 2 minutes. Cysts were finally suspended in 1-5 ml (depending upon the number of cysts) prewarmed 0.5% proteose peptone in PBS, pH 7.2, and incubated for one-half hour in a 37°C water bath. A stock proteose peptone (5% W/V) solution was prepared in distilled water, gently boiled for 10 minutes to destroy any remaining enzymes, filter sterilized, and stored at 2-5°C in small volumes. Excystation medium, prepared fresh, was made by adding 10 ml stock proteose peptone and 10 ml of 10X Phosphate Buffered Saline (PBS, 80 g NaCl, 2 g KH_2PO_4, 29 g $Na_2HPO_4 \cdot 12\ H_2O$, 2 g KCl, 1000 ml final volume) to 80 ml distilled water. This method was compared to that of Schaefer et al. (14). Both excystation methods were performed in centrifuge tubes; samples were removed and counted in a hemocytometer, using phase contrast optics at 200-400 magnification.

Between 100 and 200 empty cyst walls (ECW), partially excysted trophozoites (PET), and intact cysts (IC) were counted. The percent excystation was calculated according to the formula [ECW + PET] ÷ [IC + ECW + PET] x 100 (14).

RESULTS AND DISCUSSION

Development of exystation method for G. lamblia. A single sample from an asymptomatic human donor was used to develop serum- and enzyme-free reagents. The acid induction step (10) was retained, but improved excystation occurred when this step was increased to 45 minutes (unpublished observations). After cysts were exposed to various excystation media consisting of 0.5% phytone or proteose peptone in either HBSS or Tyrode's medium, excystation was higher (11 and 8%, respectively) than that observed (<6%) when cysts were exposed to trypsin-Tyrode's solution, phytone-TRIS, or proteose-TRIS buffer, pH 7.0.

Tyrode's medium or HBSS was next supplemented with various concentrations of phytone (0.25-2.0%). HBSS plus 2.0% phytone peptone yielded 57% excystation, whereas the other concentrations of phytone in HBSS yielded 50-55%. At phytone concentrations lower than 2%, however, the TET were nonrefractile and judged to be dead. Therefore, they were not amenable to successful separation from cyst walls by isopycnic centrifugation. In the same experiment, Tyrode's medium containing trypsin or phytone resulted in less than 21% excystation.

Further supplementation of HBSS-phytone (2.0%) with glucose (0.05% above that routinely used for HBSS) and 1.0% cysteine increased excystation to 73%. However, elimination of the extra glucose, but not cysteine or phytone, yielded 90% excystation. Controls consisting of cysts exposed to trypsin-Tyrode's medium yielded only 27% excystation. In the same experiment, lower levels of excystation resulted when cysts were exposed to HBSS (14%), HBSS-glucose (15%), HBSS plus 2.0% phytone (17%), and HBSS plus glucose and phytone (22%).

When HBSS was supplemented with only 1.0% cysteine, but not phytone,

excystation was as high as 83%. This suggested that cysteine plays an important role in the excystation process. A decrease in the concentration of cysteine to 0.1% resulted in 96% excystation, whereas, in the same experiment, elimination of cysteine by incubation of cysts in trypsin-Tyrode's medium alone reduced excystation to 57%. In all of these experiments, HBSS was supplemented with 0.15% $NaHCO_3$. This is consistent with the results of Gillin and Reiner (5) which demonstrate that G. lamblia trophozoites depend upon thiol reducing agents, such as cysteine, for survival and attachment to glass surfaces.

These results demonstrated that a complex medium containing enzymes is unnecessary and can be replaced with a simple medium consisting of a balanced salts solution, cysteine and phytone peptone. The concentration of phytone found to be most effective was slightly higher than that reported previously for the first successful in vitro excystations (2). These results suggest a minor role of some phytone constituent and a major role for L-cysteine.

Development of excystation method for G. muris. G. muris cysts were able to excyst when the above method developed for G. lamblia was used. Unfortunately, the TET died quickly and could not be adequately separated from the empty cyst walls by isopycnic centrifugation. This situation improved when proteose peptone was substituted for phytone peptone in the excystation step. Optimal appearance of the TET occurred with 0.5% proteose peptone, even though excystation was >98% with all concentrations of proteose peptone used (0.2-2.0%). Cysteine and $NaHCO_3$ were eliminated from the excystation medium after it was determined that excystation remained at >99% in the absence of these reagents. In contrast, G. lamblia cysts were incapable of excystation when proteose peptone was

substituted for phytone peptone, and showed reduced levels of excystation when cysteine was not present.

G. muris excysted (>99%) when a simple induction medium consisting of HBSS, cysteine, glutathione, and $NaHCO_3$ was used. Even though G. muris cysts were capable of >99% excystation in the presence or absence of HCl in the induction step, that reagent was omitted to avoid unnecessary exposure of cysts to it. Recently, Feely (4) reported that reducing agents were not necessary for induction of G. muris. Although reducing agents had been retained in our induction step, an experiment was performed in which the standard induction medium (HBSS, cysteine, glutathione, and $NaHCO_3$) was compared to acidified HBSS minus cysteine and glutathione (4) and HBSS to which only $NaHCO_3$ was added. While the use of the standard induction solution yielded >99% excystation, use of the latter two media resulted in lower excystation (20% and 5%, respectively). The TYI excystation medium used by Feely (4) is supplementd with serum and bile. The difference in supplementation of the excystation media could account for the decrease in excystation when reducing agents were omitted from my induction step.

Method comparison and use. Once the excystation methods for G. lamblia and G. muris were standardized, they were compared to the methods of Rice and Schaefer (10,14). For either species of cyst, both excystation procedures were performed on the same day with all samples used. As can be seen from the results (Tables 1 and 2), no apparent differences existed between the two excystation methods for both species of Giardia. Both sample-to-sample variation and extremes of percent excystation occurred, but the two methods followed similar trends as they varied. It has been previously reported (10) that asymptomatic donors of Giardia cysts yield

TABLE 1. Comparison of methods for excysting *Giardia lamblia*

Sample[a]	Cyst age (days)[b]	Mean % excystation of method (range)	
		Trypsin-Tyrode[c]	Cysteine-Phytone[d]
1	8	15 (12-17)	15 (14-17)
2	8	82 (80-84)	93 (90-96)
3	12	84 (83-85)	93 (89-96)
4	21	38 (33-43)	49 (43-54)
5	13	<1 (0-1)	2 (2)
6	14	21 (18-24)	21 (17-25)
7	15	30 (27-32)	19 (18-20)

[a]Samples 1-4 were from the same asymptomatic donor, collected at different times. Samples 5-7 were from different asymptomatic donors.

[b]Time from cyst purification to day of excystation.

[c]Method of Rice and Schaefer (11). Results were the average of two trials.

[d]Method described in text for *G. lamblia*. Results were the average of two trials. All samples were excysted on the same day.

TABLE 2. Comparison of methods for excysting *Giardia muris*

Cyst age (days)[a]	Mean % excystation of method (range)	
	Trypsin-Tyrode[b]	Proteose peptone[c]
1	84 (80-89)	91 (89-94)
2	91 (90-97)	89 (88-90)
3	91 (90-92)	89 (88-90)

[a]Time from cyst purification to excystation. The same batch of cysts was used for each day.

[b]Method of Schaefer *et al.* (15). Results were the average of three trials.

[c]Method described in text for *G. muris*. Results were the average of 3 trials.

lower excystation percentages than symptomatic carriers. However, data reported here (Table 3) show that the mean excystation of cysts from asymptomatic carriers (56% + 11% standard error) was similar to that of cysts from symptomatic donors (50% + 13% SE). This discrepancy between the previously reported data and that presented here is not surprising when data from the same donor is examined. Cysts from donors A and B (Table 3) showed extremes of percent excystation when different samples from the same donor and cysts of various ages from the same specimen were compared. Also, samples 1-4 (Table 1) from the same donor exhibited variable excystation levels. Cysts from several symptomatic carriers (Table 3) showed wide excystation variability.

The methods reported here which included the incorporation of phytone or proteose peptone and cysteine into HBSS were found to be simpler than those previously published. The latter procedures use complicated media such as HSP-3 (2) or TYI (4) and incorporate crude trypsin (10,14), bile (4,7) or whole serum (1,2,4,7) into the excystation medium. The preparation of these media is time-consuming and exposes the trophozoites and cysts to such undesirable reagents as trypsin, whole serum, or bile. For G. lamblia and G. muris cysts, it was shown that the excystation process did not require the use of serum, specific enzymes, or bile. A heat inactivated protein or protein digest such as phytone peptone, but not proteose peptone, plus a reducing agent, such as L-cysteine, were necessary in the excystation medium for G. lamblia. G. muris does not require a reducing agent such as L-cysteine in its excystation medium.

TABLE 3. *Giardia lamblia* cysts from symptomatic and asymptomatic human donors excysted utilizing a cysteine and phytone-containing medium[a]

Donor[b]	Sample	Cyst age (days)[c]	Mean % excystation[d]	No. of trials (n); range
A	1	1	93	n = 1
	1	2	90	n = 1
A	2	11	17	n = 3; 15-19
	2	13	1	n = 3; 1-2
A	3	20	96	n = 3; 95-98
B	1	2	53	n = 3; 41-63
	1	4	75	n = 2; 74 & 76
	1	8	4	n = 3; 3-5
C	1	1	21	n = 1
D	1	2	89	n = 1
E	1	4	78	n = 1
F	1	6	95	n = 1
G	1	6	93	n = 1
H	1	8	61	n = 1
I	1	10	9	n = 2; 7 & 10
J	1	12	79	n = 1
K	1	15	75	n = 1
L	1	15	31	n = 8; 25-40
M	1	15	8	n = 2; 6 & 10
N	1	16	27	n = 1
O	1	17	93	n = 3; 92-93

[a]Method described in text for *G. lamblia*.

[b]A and B were asymptomatic carriers and C through O were symptomatic donors.

[c]Time from cyst purification to excystation.

[d]At least 200 objects (IC, TET, PET) were counted per trial.

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